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D quadrant specification in the leech *Helobdella*: Actomyosin contractility controls the unequal cleavage of the CD blastomere

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ABSTRACT

The unequal division of the CD blastomere at second cleavage is critical in establishing the second embryonic axis in the leech *Helobdella*, as in other unequally cleaving spiralians. When CD divides, the larger D and smaller C blastomeres arise invariantly on the left and right sides of the embryo, respectively. Here we show that stereotyped cellular dynamics, including the formation of an intercellular blastocoel, culminate in a morphological left–right asymmetry in the 2-cell embryo, which precedes cytokinesis and predicts the chirality of the second cleavage. In contrast to the unequal first cleavage, the unequal second cleavage does not result from down-regulation of one centrosome, nor from an asymmetry within the spindle itself. Instead, the unequal cleavage of the CD cell entails a symmetric mitotic apparatus moving and anisotropically growing rightward in an actomyosin-dependent process. Our data reveal that mechanisms controlling the establishment of the D quadrant differ fundamentally even among the monophyletic clitellate annelids. Thus, while the homologous spiral cleavage pattern is highly conserved in this clade, it has diverged significantly at the level of cell biological mechanisms. This combination of operational conservation and mechanistic divergence begins to explain how the spiral cleavage program has remained so refractory to change while, paradoxically, accommodating numerous modifications throughout evolution.

Introduction

The distinctive mode of early development known as spiral cleavage is characteristic of disparate invertebrate taxa, including annelids, molluscs, polyclad flatworms and other, less-speciose phyla all belonging to the super-phylum Lophotrochozoa (Dunn et al., 2008; Giribet, 2008; Halanych et al., 1995). Spiralian development therefore offers unique opportunities for examining how the evolution of divergent body plans is achieved by developmental innovations within a conserved embryological framework.

One example of evolutionary flexibility within the spiral cleavage program is D quadrant specification (Freeman and Lundelius, 1992; Henry and Martindale, 1999). In spiral cleavage, the first two cell divisions are roughly meridional with respect to the animal–vegetal axis, establishing four embryonic quadrants (A–D). By convention, D is defined as the dorsal quadrant, which produces bilaterally symmetric trunk mesoderm and ectoderm (Dorresteijn, 2005; Lambert, 2008; Shimizu and Nakamoto, 2001; van den Biggelaar and Guerrier, 1983; Verdonk and van den Biggelaar, 1983). Thus, selecting one quadrant to be the D quadrant is fundamental to forming the second embryonic axis.

Although D quadrant specification is homologous among spiralians, significant variations in this process have evolved (Freeman and Lundelius, 1992). In equally cleaving species, the first two rounds of mitosis give rise to four blastomeres of equal developmental potential, and typically of equal size; only later is one of the quadrants selected stochastically to adopt the D fate (Arnolds et al., 1983; Martindale et al., 1985; van den Biggelaar and Guerrier, 1979). In unequally cleaving species, the D quadrant is established at the 4-cell stage by two unequal cleavages that segregate developmental determinants to one quadrant; typically the D blastomere is larger than the A, B and C blastomeres (Astrow et al., 1987; Clement, 1952; Dorresteijn et al., 1987). Equal cleavage is thought to be the ancestral state for spiralians, and unequal cleavers occur on various branches of the phylogenetic tree relating spiralian taxa. This suggests that changes from equal to unequal division have occurred multiple times and thus that D quadrant specification via unequal division is not homologous across spiralians (Freeman and Lundelius, 1992; Henry, 1986; Henry et al., 2006).

Evidence regarding intrinsic and extrinsic factors controlling unequal cell divisions in spiralian embryos have come from diverse annelids and molluscs (Dorresteijn, 2005; Goulding, 2003; Hejnol and Pfannenstiel, 1998; Inoue and Dan, 1987; Lambert and Nagy, 2001; Luetjens and Dorresteijn, 1998a; Mescheryakov, 1976; Schneider and Bowerman, 2007; Shibazaki et al., 2004; Shimizu et al., 1998; Zhang and Weisblat, 2005). To understand how a process such as D

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quadrant specification evolved, we must compare the underlying cell biological and molecular mechanisms in various species whose phylogenetic relationships are also understood. Clitellate annelids, a well-accepted monophyletic group comprising leeches (Hirudinea) and oligochaetes (Erseus and Kallersjo, 2004; Siddall and Borda, 2003; Siddall and Burreson, 1998), provide a number of experimentally tractable species for making such comparisons (Dohle, 1999; Shankland and Savage, 1997; Shimizu and Nakamoto, 2001; Weisblat, 2007). The monophyly of the clitellates allows us to make evolutionary comparisons between homologous cleavage patterns.

Detailed knowledge of clitellate embryogenesis comes primarily from studies of glossiphoniid leeches of the genera *Helobdella* and *Theromyzon* (Fernández and Olea, 1982; Weisblat and Huang, 2001) and tubificid oligochaetes of the genus *Tubifex* (Shimizu, 1982a). Essentially all clitellate embryos exhibit the following features, which are therefore considered homologous within this group. Prior to first cleavage, cytoplasmic rearrangements form domains of yolk-deficient cytoplasm, called teloplasm, that are rich in organelles and polyadenylated mRNA (Fernández et al., 1990). Teloplasm is segregated to the D quadrant and contains factors necessary for the formation of stem cells (teloblasts) that make segmental ectoderm and mesoderm (Astrow et al., 1987; Ishii and Shimizu, 1997; Nelson and Weisblat, 1991, 1992). The first cleavage results in a smaller AB cell and a larger CD cell, which inherits teloplasm. At second cleavage the CD cell divides to form a smaller C cell and a larger D cell, which inherits teloplasm. Thus, the unequal first and second cleavages establish the axes of the adult by segregating teloplasm exclusively to the D quadrant at the 4-cell stage (Weisblat et al., 1999).

Although the clitellate cleavage pattern is highly conserved, it has been shown that the mechanisms controlling teloplasm formation (Astrow et al., 1989; Shimizu, 1982b) and the unequal first cleavage (Ishii and Shimizu, 1997; Ren and Weisblat, 2006) are divergent between *Helobdella* and *Tubifex*. Specifically, teloplasm formation is a microtubule-dependent process in *Helobdella* and a microfilament-dependent process in *Tubifex* (Astrow et al., 1989; Shimizu, 1982a,b). Also, in *Tubifex*, the unequal first cleavage involves a non-duplicating, maternally-derived centrosome so that the mitotic apparatus is monoastral and highly asymmetric throughout first cleavage (Ishii and Shimizu, 1995, 1997; Shimizu, 1982a,b), whereas in *Helobdella*, the centrosome is paternally-derived and duplicates early in the first cell cycle. The resultant biastral mitotic apparatus (MA) in *Helobdella* is symmetric through early metaphase; then, one centrosome is down-regulated, followed by the partial collapse of the associated aster, which renders the MA asymmetric and leads to the unequal first cleavage (Ren and Weisblat, 2006). These studies demonstrated that despite the fact that teloplasm formation and unequal first cleavage are homologous events in clitellates, the cell biological mechanisms controlling them are different. Comparing the mechanisms controlling D quadrant specification in *Helobdella* versus *Tubifex* therefore gives us clues about the evolutionary constraints and permissions of the spiral cleavage program.

The work presented here extends these comparisons by addressing the mechanisms underlying the inequality and chirality of the CD cleavage in *Helobdella* (Fig. 1). We show that the unequal cleavage of the CD cell entails an intimate connection between the mitotic apparatus and the cortex at the interface between the AB and CD cells. The CD spindle is symmetric and biastral and attaches via both asters to the cortex surrounding an intercellular blastocoel that forms during the 2-cell stage. The CD MA initially resides equidistant from the edges of the CD cell and subsequently becomes displaced toward the right side of the cell beginning in metaphase, inducing an eccentrically located cytokinetic furrow. Pharmacological perturbation of the microtubule and actomyosin cytoskeletons revealed that: 1) the intimate connection between the spindle poles and the basolateral cortex is necessary for proper spindle orientation and 2) the rightward movement of the mitotic apparatus is controlled by actomyosin contractility. We discuss the evolutionary implications of these findings in the context of D quadrant specification in relation to *Tubifex* and other spiralians.

Methods and materials

Animal culture and embryonic timing

Embryos were obtained from a permanent laboratory culture of *Helobdella* sp. (Austin), originating from Austin, Texas (Bely and Weisblat, 2006). *Helobdella* zygotes are deposited one by one, so each clutch (20–100 zygotes) is slightly asynchronous. For precise timing, developmental events are designated as occurring at a particular time after zygote deposition (AZD) (Weisblat and Huang, 2001; Yazdani and Terman, 2006) at 23 °C. We grouped embryos that had begun first cleavage (as judged by the first deformation of the plasma membrane) within a 5-minute window (usually 3–10 embryos) and defined this group as 265 ± 2.5 min AZD. Embryos were cultured at 23 °C in Htr medium (Blair and Weisblat, 1984).

Immunohistochemistry

Fixation and immunostaining were carried out as for the zygote (Ren and Weisblat, 2006). Mouse monoclonal antibody against beta-tubulin (Sigma, T-0198 clone number D66) was used at 1:1000; rabbit polyclonal antibody against sea urchin tubulin was a gift of the Cande lab at U.C. Berkeley and was used at 1:25; rabbit polyclonal antibody against gamma-tubulin (Sigma, T-3559) was used at 1:2000; rabbit polyclonal antibody against actin (Sigma, A2066) was used at 1:50; mouse monoclonal antibody against histone H1 (Chemicon, MAB052) was used at 1:2000; rabbit polyclonal antibody against myosin light chain (phospho S20; AbCam, ab2480) was used at 1:500. Alexa fluor-labeled fluorescent secondary antibodies (Molecular Probes) were used at 1:500; Cy3- and Cy5-labeled antibodies (Jackson Immunosciences) were used at 1:800; Cy2-labeled antibodies (Jackson Labs) were used at 1:50. Following immunohistochemistry, embryos were dehydrated through an ethanol series and cleared in 3:2 benzyl benzoate:benzyl alcohol (BBBA) for confocal microscopy.

Drug treatments

Primary stocks of nocodazole (20 μM, Sigma, M1404), cytochalasin D (25 mM, Sigma, C8273), and blebbistatin (100 mM, Toronto Research Chemicals, B592500) were made in DMSO and stored at −20 °C. Primary stock of ML-7 (300 μM, Calbiochem, 475880) was made in DMSO and stored at 4 °C. Working stocks were prepared immediately prior to use by dilution with Htr; control embryos were incubated in matching dilutions of DMSO (0.2–0.05%). For all drug treatments, each group of embryos undergoing their first cleavage within a 5-minute window (see above) was split and immediately immersed in drug or control solutions and incubated at room temperature throughout the 2-cell stage for live observations or subsequent fixation and immunohistochemistry. Three or more experiments were performed for each drug treatment.

Microscopy, imaging software and image analysis

Live embryos were viewed with a dissecting microscope under fiber optic illumination. Images were captured with a digital camera (Nikon cool pix) mounted on the dissecting microscope. Immunostained and BBBA-cleared embryos were examined by laser scanning